#10 Allowable 4/1/03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Ream et al.

Art Unit: 1649

Application No. 09/434,837

Filed: November 4, 1999

For: PLANTS HAVING ENHANCED RESISTANC TO GALL AND METHODS AND COMPOSITIONS FOR PRODUCING SAME

Stuart - Punch holes + attach this to the case - Betz

Examiner: Stuart F. Baum

Date: January 2, 2003

COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

DECLARATION OF L. WALTER REAM, JR., Ph.D. UNDER 37 C.F.R. § 1.132

I, L. WALTER REAM, JR., Ph.D. declare as follows:

1. I am an inventor of and have read and understand U.S. Patent Application No. 09/434,837 entitled PLANTS HAVING ENHANCED RESISTANCE TO GALL AND METHODS AND COMPOSITIONS FOR PRODUCING SAME, and all amendments in that application through today's date. In addition, I have read and understand the following reference: Hiroyasu *et al.* (Kokai Number (1993) 68574).

2. A copy of my *curriculum vitae* is attached hereto as **Exhibit B**. At present, I, L. Walter Ream, Jr., Ph.D., hold an academic position as Professor of Microbiology and Director of the Genetics Program at Oregon State University.

3. Further, I understand that Claims 1-16 and 25-40 are currently pending in the application, and that Claims 1-7, 9-14, and 16 are rejected as allegedly anticipated (under 35 U.S.C. 102(b)) by the teachings of Hiroyasu *et al.*

4. In my experience, as a researcher well versed in the study of RNA silencing, bacterial genetics, and plant-microbe interactions, the segment of untranslatable RNA that is effective in post-transcriptional gene silencing (PTGS) must be determined empirically for each RNA of interest. In addition to our personal experience, this has been recognized by others of ordinary skill of the art (for instance, Mary K. Montgomery, Ph.D., personal communication).

5. I have found that PTGS is a highly effective method of suppressing *iaaM* expression in plants susceptible to crown gall tumors caused by the transformation of plant cells with *Agrobacterium tumefaciens*. I have accomplished this by designing a transgene that expresses an untranslatable sense-strand *iaaM* RNA. The untranslatable sense-strand *iaaM* RNA includes an 1800 nucleotide long portion of the *iaaM* RNA. In order to generate the untranslatable sense-strand *iaaM* RNA, residues 1 through 1802 of wild-type *iaaM* were engineered to contain a TGA stop sequence at the third codon, as well as the deletion of two bases immediately following the third codon, yielding a construct that is 1800 base pairs long (*iaaM*-stop). Tobacco plants homozygous for the *iaaM*-stop transgene silenced *iaaM* expression in 56% of the plant lines (Figure 3, **Exhibit C**), thus the *iaaM*-stop transgene is highly effective at preventing crown gall tumor growth.

6. I have found that particular nucleotide sequences within the 1800 nucleotide long fragment of the *iaaM* RNA may be more effective at generating double-stranded RNA molecules that are capable of suppressing *iaaM* expression in plants than other *iaaM* nucleotide sequences. To examine sequence requirements for *iaaM* silencing, I tested the ability of three different 600 base pair fragments which included residues 1-600, 600-1200, or 1200–1800 (derived from the highly effective 1800 base pair transgene) to silence *iaaM* (Figures 8 and 9, **Exhibit C**). Potato disks developed wild-type tumors, when co-inoculated with *Agrobacterium tumefaciens* and the 600 base pair constructs, as frequently as the disks that were co-inoculated with *Agrobacterium tumefaciens* and a vector only construct, indicating that these constructs do not suppress *iaaM* expression. This experiment was repeated in *Kalanchoe daigremontiana* stems with similar results, confirming that the 600 base pair *iaaM* fragments are incapable of silencing *iaaM* expression. Furthermore, truncations of 200 base pairs (yielding a construct including residues at positions 1 through 1615) and 400 base pairs (yielding a construct including residues at

positions 1 through 1413) from the 3' end or 200 base pairs (yielding a construct including residues at positions 189 through 1813) from the 5' end of the full-length *iaaM* transgene abolished its ability to silence *iaaM*. However, a 400 base pair truncation (yielding a construct including residues at positions 382 through 1813) at the 5' end of the transgene partially silenced the wild-type *iaaM* oncogene (Figures 8 and 10, **Exhibit C**). Since only one out of eight constructs had a significant impact on reducing oncogene expression, and a second construct had an intermediate effect, I believe that the silencing efficiency of a double-stranded RNA molecule varies with the presence and/or absence of particular target sequences. Thus, the ability to identify regions within a gene that contain the most effective suppressive RNA sequences must be determined empirically for each gene.

7. Based on the data presented above, I believe that I would not have been guided by Hiroyasu *et al.* to use an 1800 base pair fragment or, moreover, that a fragment of that size would have a superior effect on silencing *iaaM* expression than a smaller fragment. Hiroyasu *et al.* describes a 697 base pair *iaaM* fragment (residues 143 through 840) that is reportedly effective in preventing tumor growth in tobacco plants transformed with *Agrobacterium tumefaciens*. However, I have generated evidence that three different 600 base pair constructs (1-600, 600-1200, and 1200-1800) were not capable of silencing *iaaM* (Figure 9, **Exhibit C**), thus having an *iaaM* fragment of approximately the same size is not sufficient to inhibit tumor growth in plants. Moreover, a 1400 base pair construct (1-1413) that contains the Hiroyasu *et al.* 697 base pair fragment was not capable of silencing *iaaM* (Figure 10, **Exhibit C**). Thus, merely having a sequence containing the 697 base pair fragment is therefore not sufficient to prevent tumor growth in plants. Furthermore, Hiroyasu *et al.* does not compare the effect of the 697 base pair fragment to *iaaM* fragments of other sizes. One of ordinary skill in the art would not be able to predict, based solely on the Hiroyasu *et al.* reference, and without further experimentation, which portion of the *iaaM* sequence would be most effective at PTGS.

8. I have also found that the *iaaM* transgene, which is derived from an octopine-type Ti plasmid, will silence the *iaaM* gene from a nopaline-type Ti plasmid. The octopine and nopaline-type *iaaM* genes are similar but not identical. In fact, they have 94% sequence identity (**Exhibit A**, also submitted with the Amendment and Response to the Office action on December

6, 2002). This experiment demonstrates, therefore, that the transgene that induces the silencing does not have to be identical to the target gene to be silenced.

9. It is my experience that the introduction of a single stop codon in the *iaaM* nucleic acid sequence is sufficient to silence *iaaM* expression. The introduction of a double mutation (a stop codon in addition to a frame-shift) is a precautionary method routinely used by geneticists solely to reduce the likelihood, in a transformed crop plant, of a reversion of a transgene into a functional oncogene. For example, each mutation by itself may revert at a low (<1 in 1,000,000) frequency, whereas both mutations would revert much less often (<1 in 1,000,000,000,000).

10. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

Date: January 2, 2003

Lloyd Walter Ream Jr.
L. Walter Ream, Jr., Ph.D.